IL-21 signaling is critical for the development of type I diabetes in the NOD mouse

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IL-21 is a pleiotropic type I cytokine that shares the common cytokine receptor γ chain and plays important roles for normal Ig production, terminal B cell differentiation to plasma cells, and Th17 differentiation. IL-21 is elevated in several autoimmune diseases, and blocking its action has attenuated disease in MRL/lpr mice and in collagen-induced arthritis. The diabetes-associated *Idd3* locus is at the *Il2*/*Il21* locus, and elevated IL-21 was observed in the nonobese diabetic (NOD) mouse and suggested to contribute to diabetes by augmenting T cell homeostatic proliferation. To determine the role of IL-21 in diabetes, *Il21r*-knockout (KO) mice were backcrossed to NOD mice. These mice were devoid of lymphocytic infiltration into the pancreas, and only 1 of 20 animals had an elevated glucose compared with 60% of NOD mice on a wild-type (WT) background. Although TCR and Treg-related responses were normal, these mice had reduced Th17 cells and significantly higher levels of mRNAs encoding members of the Reg (regenerating) gene family whose transgenic expression protects against diabetes. Our studies establish a critical role for IL-21 in the development of type I diabetes in the NOD mouse, with obvious potential implications for type I diabetes in humans.

Reg genes | Th17 cells

Human type I diabetes is an autoimmune disease that results from the autoreactive destruction of pancreatic β cells and subsequent loss of insulin production (1). Nonobese diabetic (NOD) mice develop a similar disease and serve as a model system for studying the mechanisms involved in the initiation and propagation of the autoimmune response (2). Although it has been established that β islet cell destruction is initiated by proinflammatory $CD4^+$ and $CD8^+$ T cells infiltrating the pancreas, the molecular mechanisms and cytokine pathways that control this process are not fully understood.

IL-21 is a type I cytokine (3) that is the most recently discovered member of the family of cytokines that share the common cytokine receptor γ chain, γ_c (4). γ_c is also shared by IL-2, IL-4, IL-7, IL-9, and IL-15 and is mutated in humans with X-linked severe combined immunodeficiency (XSCID) (5). IL-21 is produced most abundantly by $CD4^+$ T cells, including Th17 lineage cells (6–8) and natural killer T (NKT) cells (9), and is known to act on both lymphoid and nonlymphoid target cells, thus controlling both innate and adaptive immune responses (4). IL-21 is critical for normal Ig production (10), can cooperatively drive expansion of $CD8^+$ T cells (11), can activate NK cells (12), and drives terminal B cell differentiation into plasma cells (13). IL-21 has been implicated as playing a role in the development of B cell-mediated lupus-like autoimmune disease based on studies in the BXSB-*Yaa* (13), *Sanroque* (14), and MRL/lpr mice (15), and treatment with a soluble IL-21R-Fc fusion protein was shown to lead to a partial decrease in disease severity in the MRL/lpr mice (15) and collagen-induced arthritis (16). In experimental allergic encephalitis (EAE), a model for multiple sclerosis, IL-21 was shown to be critical for the development of the inflammatory Th17 lineage for the pathogenesis of the disease (7, 8).

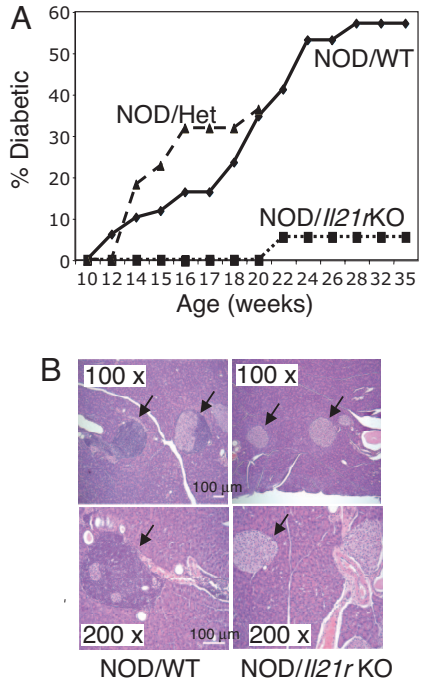

Fig. 1. NOD/*Il21r*-KO mice do not develop diabetes or insulitis. (*A*) *Il21r*-KO mice were bred onto the NOD background for seven generations. Blood glucose levels were measured weekly in 51 NOD/WT and 20 NOD/*Il21r*-KO littermates starting at the age of 10 weeks, and glucose levels >250 mg/dl were scored as diabetic. Twenty-two NOD/$Il21r^{+/-}$ mice were also analyzed up to 20 weeks of age, at which point they were killed. In addition, blood glucose levels were measured in all subsequently killed KO mice used in this work and were found to be in the normal range (<200 mg/dl). (*B*) Representative pancreatic sections from 12-week-old NOD/WT and NOD/*Il21r*-KO mice showing typical islet infiltration only in the WT pancreas (*Left*). (Magnification: *Upper*, ×100; *Lower*, ×200.) Four additional NOD/WT and NOD/*Il21r*-KO mice were examined, with results similar to those shown in the figure. H&E stain was used. (Scale bars: 100 μm.)

The interest in IL-21 as a potential mediator of autoimmune diabetes stemmed from the fact that the *Il21* gene lies within the *Idd3* locus that is associated with disease in the NOD mouse model of type I diabetes, and levels of IL-21 and expression of IL-21R were reported to be elevated in the NOD mouse and to contribute to homeostatic expansion that was suggested to correlate with disease (17). Although IL-21 subsequently was reported to not be the critical component of the *Idd3* locus (18),

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Conflict of interest statement: R.S. and W.J.L. are inventors on patents and patent applications related to IL-21.

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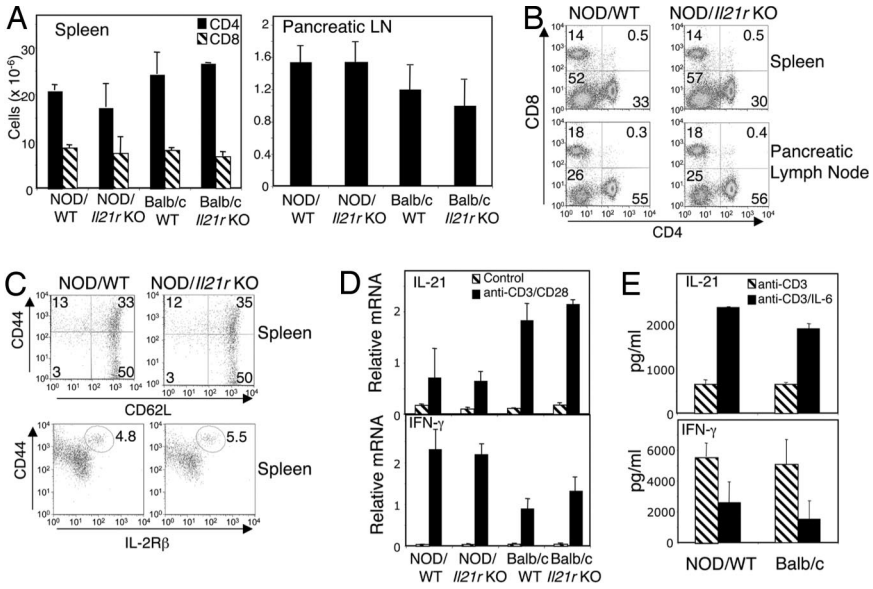

Fig. 2. Normal lymphoid cellularity and activation markers in NOD mice. (*A* and *B*) Splenic T cell populations and pancreatic lymph node cellularity were quantified by flow cytometry ($n = 12$ for NOD/WT, $n = 12$ for NOD/*Il21r*-KO, $n = 6$ for BALB/c WT, $n = 6$ for BALB/c*Il21r*-KO); mean ± SEM. (*A*) Tabulation of data. (*B*) Representative flow cytometric profiles. (*C*) Splenic T cells were analyzed by flow cytometry for the activation markers CD62L, CD44, and IL-2Rβ. Representative FACS profiles from one of three independent experiments are shown. (*D*) $CD4^+$ T cells were isolated and stimulated either with or without anti-CD3/anti-CD28 for 6 h, at which time IL-21 and IFN-γ mRNA were analyzed by quantitative RT-PCR. The results were normalized relative to expression of *Rpl7*. Shown are mean ± SD from three mice in each group; results are representative of three independent experiments. (*E*) $CD4^+$ T cells (2×10^6 per ml) were stimulated with anti-CD3/anti-CD28 either in the presence or absence of IL-6 for 48 h, at which time supernatants were assayed by ELISA for either IL-21 or IFN-γ. Shown is the mean ± SD for results from three mice in each group.

the pleiotropic actions of IL-21 on multiple lineages potentially involved in the development of the autoreactive inflammatory immune response in diabetes are consistent with the possibility of IL-21 contributing to this disease through its actions on immune cells or cytokine networks.

In this work, we evaluated the role of IL-21 in the initiation of the diabetic changes that occur spontaneously in the NOD mouse. We find that deletion of IL-21 signaling leads to the almost complete abrogation of disease development. Examination of lymphoid and cytokine profiles in prediabetic NOD/wild-type (WT) and NOD/*Il21r*-knockout (KO) mice revealed that Th17 responses are deficient in mice lacking IL-21R expression even at an early time point. These studies establish an essential role for IL-21 in the pathogenesis of diabetes in the NOD mouse.

Results

IL-21 Is Required for the Development of Diabetes. To examine the potential role of IL-21 in the development of type I diabetes, we produced NOD/*Il21r*-KO mice by backcrossing our *Il21r*-KO mice (10) with the NOD/LtJ strain that spontaneously develops insulitis followed by hyperglycemia and full-blown diabetes beginning at ≈12 weeks of age. To track the development of diabetes, blood glucose levels were monitored in a cohort of NOD/WT and NOD/*Il21r*-KO littermates for a period of 9 months, and animals were considered diabetic if blood glucose was consistently >250 mg/dl. Strikingly, NOD mice on the *Il21r*-KO background were nearly completely protected from development of diabetes, with disease in only 1 of 20 mice by 9 months compared with 60% of the 51 NOD/WT littermates (Fig. 1*A*). Analysis of blood glucose levels in NOD/$Il21r^{+/-}$ heterozygous littermates was also performed for ages up to 20 weeks of age, at which point 8 of 22 (36%) had developed diabetes, an incidence similar to the NOD/WT mice at that age (Fig. 1*A*).

To assess whether resistance to the development of diabetes was accompanied by reduced pancreatic islet inflammation, pancreatic sections from 12-week-old NOD/WT and NOD/*Il21r*-KO mice were examined. Histological results showed that the NOD/WT pancreas in this age group had typical infiltration in the majority of examined islets, whereas the NOD/*Il21r*-KO islets were devoid of inflammatory infiltration (Fig. 1*B*). These results indicate that the lack of IL-21 signaling almost completely prevents diabetes as a result of the early inhibition of the development of insulitis.

Normal T Cell Populations and Cytokine Production in NOD/*Il21r*-KO Mice. To explore the possibility that alterations in peripheral T cell numbers or function might play a role in the resistance of the NOD/*Il21r*-KO mice to development of disease, we assessed $CD4^+$ and $CD8^+$ T cell numbers in the splenic compartment and in the draining pancreatic lymph nodes. Unexpectedly, in contrast to a previous report that lymphopenia occurs in NOD mice (17), we reproducibly found that NOD/WT and NOD/*Il21r*-KO mice had similar splenic (Fig. 2*A Left*) and pancreatic lymph node (Fig. 2*A Right*) T cell numbers compared with WT and *Il21r*-KO mice on the BALB/c background. Thus, IL-21 does not appear to be acting to mediate homeostatic proliferation in NOD mice. Furthermore, a comparison of $CD4^+/CD8^+$ T cell ratios in spleen and pancreatic lymph nodes revealed no obvious differences in the NOD/WT and NOD/*Il21r*-KO mice (Fig. 2*B*) and no differences in levels of activation markers such as CD62L, CD44, or IL-2Rβ (Fig. 2*C*).

Because the NOD/*Il21r*-KO mice had such a dramatic decrease in diabetes compared with NOD/WT mice, we explored whether TCR-stimulated $CD4^+$ T cells from disease-prone NOD mice produced increased levels of IL-21. Upon *ex vivo* stimulation for 6 h with anti-CD3 + anti-CD28, IL-21 mRNA levels in NOD $CD4^+$ T cells were approximately half the level seen after similar stimulation in BALB/c $CD4^+$ T cells (Fig. 2*D Upper*). We also examined IL-21 protein levels in NOD or BALB/c T cells stimulated either with either anti-CD3/anti-CD28 alone or anti-CD3/anti-CD28 plus IL-6, which potently up-regulates IL-21 (6), and we found at most a modest difference in the secreted IL-21

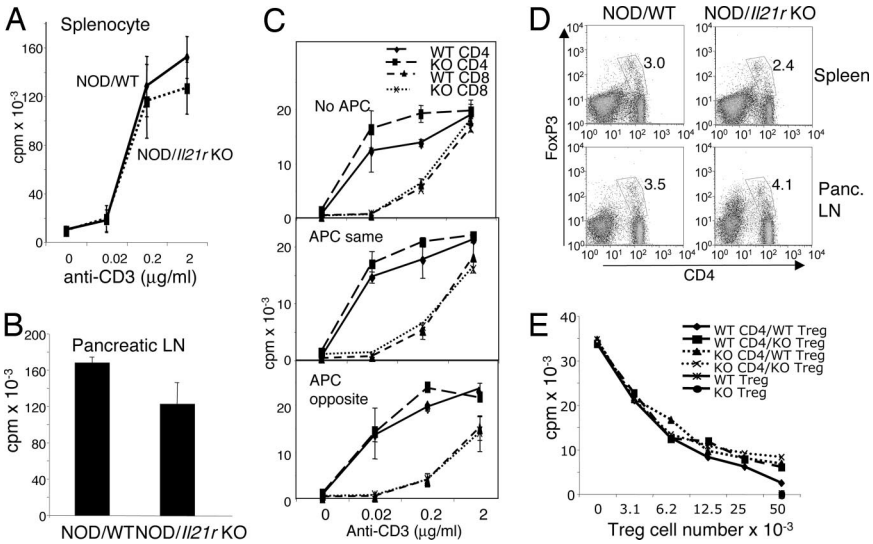

Fig. 3. T cell proliferative responses and Treg cell function are normal in NOD/*Il21r*-KO mice. (*A* and *B*) Splenocytes (*A*) or pancreatic lymph nodes (*B*) (10^6 per ml) were cultured in microwells with plate-bound anti-CD3 at the indicated concentrations for splenocytes and at 2 μg/ml for pancreatic lymph nodes for 48 h and were pulsed with [^{3}H]thymidine for the last 8 h. No significant difference was observed in the proliferation of NOD/WT vs. NOD/*Il21r*-KO splenocytes or pancreatic lymph node cells. Shown is a representative experiment, with error bars indicating the SD for a group of three mice. (*C*) Splenic CD4$^+$ or CD8$^+$ T cells from WT or KO mice were stimulated with increasing concentrations of anti-CD3 in the presence or absence of irradiated T-depleted splenic APCs from either WT or KO mice for 48 h and were pulsed with [^{3}H]thymidine for the last 8 h. Shown is a representative experiment, with error bars indicating the SD for a group of three mice. (*D*) Splenic and pancreatic LN Treg cells were quantitated by intracellular staining with FoxP3 Ab. Representative staining profiles are shown. Average percentages of FoxP3$^+$ pancreatic lymph nodes of nine mice from three different experiments were as follows: NOD/WT, 3.6 $\pm$ 0.3; NOD/*Il21r*-KO, 3.9 $\pm$ 0.4. (*E*) CD4$^+$ NOD/WT or NOD/*Il21r*-KO cells (5×10^4 per well) were cultured with increasing numbers of purified splenic Tregs from either NOD/WT or NOD/*Il21r*-KO mice in the presence of irradiated syngeneic T-depleted APCs and anti-CD3 for 3 days with [^{3}H]thymidine labeling during the last 8 h of culture. Shown is mean $\pm$ SD for results from three mice per group from a representative experiment.

protein levels by NOD versus BALB/c T cells (Fig. 2*E Upper*). *Ex vivo* IFN-γ mRNA levels produced by NOD CD4$^+$ T cells were higher than those produced by BALB/c T cells, in keeping with the known role for IFN-γ in inflammatory responses (Fig. 2*D Lower*), but total IFN-γ protein secretion by 48 h was equivalent in response to anti-CD3/CD28 and was repressed by the addition of IL-6 in both NOD and BALB/c CD4$^+$ T cells (Fig. 2*E Lower*). Thus, differences in disease susceptibility cannot be explained by strain-specific differences in IL-21 protein production, and resistant NOD/*Il21r*-KO mice do not have alterations in IFN-γ levels that would account for their resistance to disease.

Proliferative Responses and Treg Activity Are Not Altered in NOD/*Il21r*-KO Mice. A possible explanation for the disease resistance of the NOD/*Il21r*-KO mice could come from an alteration in the inherent antigen responsiveness of T cells from these mice or an increase in either the number or functional activity of Treg cells in the lymphoid compartment. Proliferative responses of NOD/WT and NOD/*Il21r*-KO T cells to anti-CD3 were similar in the spleen with at most a trend toward a slight decrease in the proliferation of NOD/*Il21r*-KO T cells at high concentrations of anti-CD3 (Fig. 3*A*). A modest decrease in proliferation was seen in the NOD/*Il21r*-KO pancreatic lymph nodes (Fig. 3*B*). To assess proliferative responses further, CD4$^+$ or CD8$^+$ T cells from WT or KO mice were stimulated with anti-CD3 either in the absence of antigen-presenting cells (APCs) or in the presence of either WT or KO-irradiated APCs. Proliferative responses of CD4$^+$ and CD8$^+$ T cells were similar and were unaffected by the presence of either WT or KO APCs (Fig. 3*C*). The number of Treg cells as assessed by FoxP3 expression was also similar between the WT and the KO mice in both the spleen and pancreatic lymph nodes (Fig. 3*D*). Although the number of Treg cells were similar, it was nevertheless possible that there was a difference in their ability to suppress T cell proliferative responses. However, when Tregs from NOD/WT and NOD/*Il21r*-KO spleens were compared for their ability to suppress proliferative responses of CD4$^+$ NOD/WT T cells, there was no statistically significant difference in their functional activity (Fig. 3*E*), consistent with the relatively similar T cell proliferative responses seen in Fig. 3*A*. In addition, there was no difference in the ability of WT CD4$^+$ and KO CD4$^+$ T cells to be suppressed by Tregs.

Th17 Function Is Reduced in NOD/*Il21r*-KO Mice. Although the role of IL-17-producing cells in the development of diabetes has not been definitively addressed, it has been suggested that these cells may contribute to the pathogenicity of autoreactive T cells in the disease (19, 20). The recently elucidated role for IL-21 in the development of Th17 cells (6–8) therefore suggested that Th17 cells might play a role in the IL-21-mediated development of diabetes, and increased IL-17 levels have been identified in the later phase of progression from insulitis to active diabetes (21). We investigated the development of Th17 cells in the prediabetic stage in NOD/WT and NOD/*Il21r*-KO mice. CD4$^+$ T cells from these mice were stimulated *ex vivo* under Th17 or Th1 polarizing conditions, and IL-17 or IFN-γ producing cells were measured. In both the pancreatic lymph node and splenic populations, the number of IL-17-producing cells detected by intracellular staining was significantly reduced in the NOD/*Il21r*-KO CD4$^+$ T cell population (Fig. 4*A*), as was the amount of secreted IL-17 detected by ELISA (Fig. 4*B*). The number of IFN-γ-producing cells was similar in the pancreatic lymph nodes, whereas those in the spleen were just slightly lower in NOD/*Il21r*-KO than in NOD/WT mice (Fig. 4*A*), and this corresponded to the secreted IFN-γ levels detected by ELISA (Fig. 4*B*). When CD4$^+$ T cells from NOD/WT or NOD/KO mice were stimulated with anti-CD3 under nonpolarizing conditions either in the presence or absence of irradiated APCs, the amount of secreted IL-17 by KO cells was significantly reduced compared with WT cells, and this

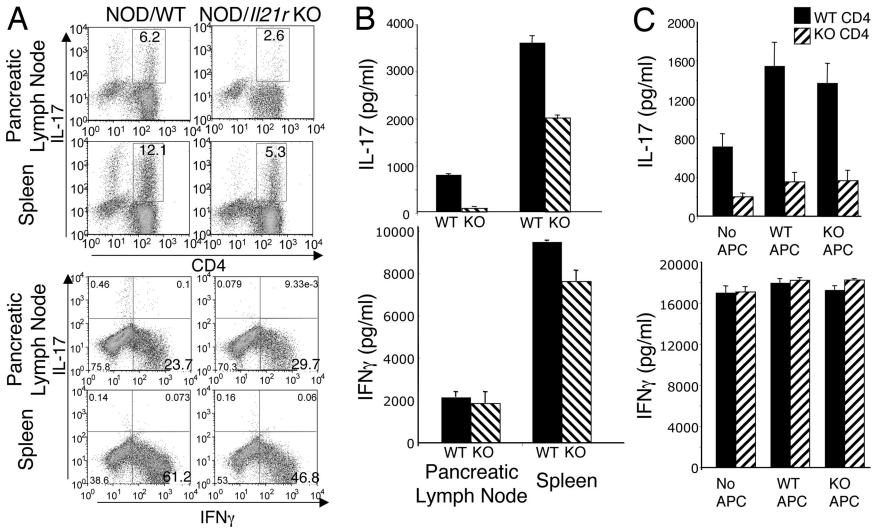

Fig. 4. IL-17 production is reduced in CD4$^+$ T cells from pancreatic lymph nodes or spleen of NOD/*Il21r*-KO mice. (*A*) T cells from pancreatic lymph node or spleen were cultured with either TGF-β (2 ng/ml) + IL-6 (2 ng/ml) (*Upper*) or with IL-12 (10 ng/ml) (*Lower*) in plates with plate-bound anti-CD3 (2 μg/ml) + anti-CD28 (1 μg/ml) for 48 h. Cells were then stimulated with PMA + ionomycin in the presence of Golgi plug for 5 h and were intracellularly stained with IL-17A and IFN-γ mAbs. Shown is a representative profile of one of three mice from an experiment with three mice in each experimental group; three similar independent experiments were performed. (*B*) Culture supernatants were collected at 48 h, and levels of secreted IL-17 and IFN-γ were measured by ELISA. Shown is mean ± SD for results from three mice per group from a representative experiment. (*C*) CD4$^+$ T cells from NOD/WT or NOD/*Il21r*-KO mice were stimulated under nonpolarizing conditions with anti-CD3 either in the absence or the presence of irradiated T-depleted splenic APC for 48 h, and culture supernatants were assayed for secreted IL-17 and IFN-γ by ELISA.

difference was seen in both the absence and presence of APCs, although secreted IL-17 was higher in general when APCs were included in the cultures (Fig. 4*C*). Equivalent levels of IFN-γ were secreted by WT and KO CD4$^+$ T cells.

Reg Proteins Are Up-Regulated in Pancreas from NOD/*Il21r*-KO Mice. Regenerating gene (*Reg*) was identified as a gene that is up-regulated in regenerating pancreatic β islet cells (22). Whereas deletion of the *Reg2* gene led to decreased proliferation of the pancreatic β islet cells, NOD mice carrying a Reg2 transgene specifically expressed in the pancreas displayed a significant delay in the onset of diabetes (23). We therefore examined the expression of two members of this family of proteins, Reg2 and PAP, in the pancreas of WT and *Il21r*-KO mice either on the NOD or on the C57BL/6 background. Significantly higher levels of both Reg2 (Fig. 5 *Upper*) and PAP (Fig. 5 *Lower*) were found in the *Il21r*-KO pancreas on both the NOD and the C57BL/6 background. Thus, both increased Reg gene expression and protection from the development of diabetes in the NOD mouse are associated with IL-21R deficiency. These results suggest that higher expression of these proteins in the absence of IL-21 may allow enhanced proliferation or regeneration of pancreatic β cells, leading to an enhanced resistance to islet destruction.

Discussion

In this work, we used the NOD mouse model and demonstrated that IL-21 plays a key role in the initiation of the early events of insulitis. Accordingly, the absence of insulitis in NOD/*Il21r*-KO mice suggests that blocking IL-21 actions may have clinical potential in the treatment of diabetes. Our experiments demonstrate that the effects of IL-21 are not the result of chronic overexpression of this cytokine in the NOD mouse because levels of IL-21 protein were similar to those in BALB/c WT mice. These data differ from those in a previous study that reported that IL-21 mRNA was up-regulated in 60% of NOD mice (17). NOD mice used in this previous study were also found to be lymphopenic, and homeostatic expansion in response to this deficit was hypothesized to be the causative factor in the development of the autoreactive response. However, we find no evidence for lymphopenia in prediabetic NOD mice, consistent with another study that compared prediabetic NOD mice with three other nonautoimmune mouse strains and found no changes in thymic or peripheral lymphocyte numbers (24).

Two previous studies have reported changes in Treg number or function in the NOD mice that could account for loss of tolerance (25, 26), whereas a third study focusing on the prediabetic phase found no difference in Treg number (24). Our

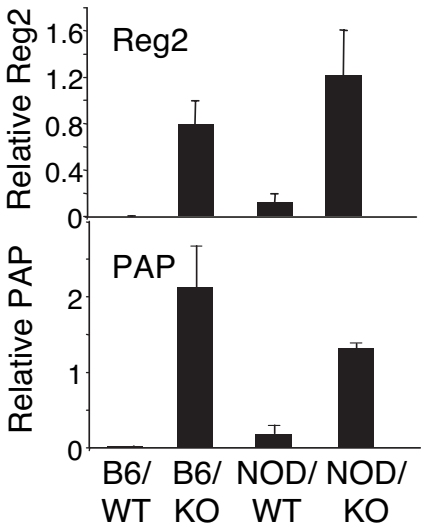

Fig. 5. *Il21r*-KO pancreas expresses higher levels of two members of the Reg family of mRNAs. Pancreatic RNA from 6- to 8-week-old WT and *Il21r*-KO mice on either the C57BL/6 or the NOD background was prepared and subjected to analysis by quantitative real-time PCR. RT-PCR revealed significantly higher levels of Reg2 or PAP mRNA in *Il21r*-KO mice on either background. Shown is the mean ± SD for three mice in each experimental group. Three similar independent experiments were performed.

experiments find no difference in either Treg number or function in NOD/WT vs. NOD/*Il21r*KO mice at a time point at which there are already striking differences in insulitis. In addition, we detected no alterations in the ability of WT and KO $CD4^+$ T cell proliferation to be suppressed by Tregs. It is conceivable that changes in Treg function might play a role later in the disease in response to a secondary systemic autoimmune response.

One of the differences between NOD/WT and NOD/*Il21r*-KO mice was in their ability to mount an IL-17 response. IL-21 plays an important role in the initiation of molecular changes that cause Th17 cell differentiation (6–8). Although IL-17 plays a major role in the inflammatory response in autoimmune diseases such as EAE, its role in autoimmune diabetes remains poorly understood. Diabetes induction can be accelerated by IL-23 treatment that leads to IL-17 induction (20), and IL-17 has been shown to increase the level of inducible nitric oxide (NO) synthase in pancreatic β cells, leading to the production of NO and subsequent β cell damage (27). Nevertheless, the effects of blocking IL-17 are variable depending on circumstances. Specifically, recent experiments (19) have demonstrated that although Th17-polarized effector T cells can transfer diabetes and that this could be blocked with antibodies to IL-17, the transfer of diabetes with nonpolarized cells could not be blocked with these antibodies. Thus, the relationship of IL-17 to diabetes in the NOD mouse remains unclear.

An interesting finding in this work was that the *Il21r*-KO pancreas expresses higher levels of two Reg family genes. Although Reg proteins are known to function as β cell trophic factors, it has also been shown that Reg2 in the pancreas can function as an autoantigen and the target of an islet-destructive process (28, 29). However, when Reg was specifically expressed as a transgene in the pancreas of NOD mice, the onset of diabetes was significantly delayed (23). Although the molecular mechanism leading to up-regulated Reg expression in the *Il21r*-KO remains to be determined, it is thus possible that the augmented Reg gene expression contributes to the protective effect from diabetes observed in the *Il21r*-KO NOD mice.

In summary, our results indicate that the development of diabetes in the NOD mouse depends on IL-21 signaling. Further investigation is clearly indicated to determine the role for IL-21 for human type I diabetes as well.

Materials and Methods

Mice. *Il21r*-KO mice were generated by homologous recombination using 129Sv ES cells that were then injected into C57BL/6 blastocysts (10). Chimeric mice were crossed with C57BL/6 mice, and heterozygous F1 progeny were crossed with BALB/c mice for four generations, at which point these mice were backcrossed for seven generations with NOD/LtJ (Jackson Laboratory) to generate NOD/*Il21r*-KO mice. Experimental animals were produced by breeding heterozygous mice, and littermates were used throughout as controls. BALB/c mice were obtained from Jackson Laboratory. All experiments were performed under protocols approved by the National Heart, Lung, and Blood Institute/National Institutes of Health Animal Care and Use Committee.

Assessment of Hyperglycemia and Diabetes. Blood glucose levels were measured by using test strips and the One Touch monitoring system. Mice were considered hyperglycemic when their blood glucose consistently rose above 250 mg/dl.

Histology. Pancreatic tissue from both NOD/WT and NOD/*Il21r*-KO mice was harvested from 12-week-old prediabetic mice, fixed in 10% formalin and embedded in paraffin, and 5-μm thickness sections were cut, stained with H&E, and analyzed by light microscopy for evidence of islet infiltration. Histopathological findings were made by random assignment of the samples, and the scores for the islet morphology and lymphocyte infiltration were then collected.

Flow Cytometric Analysis. Single-cell suspensions were prepared from spleen or pancreatic lymph nodes. Cells were surface-stained in FACS buffer (PBS containing 0.5% BSA and 0.02% azide). All antibodies for surface staining were from BD Biosciences. For intracellular staining, cells were activated with 10 ng/ml PMA and 1 μM ionomycin (both from Sigma–Aldrich) for 5 h in the presence of Golgi Plug (BD Biosciences). Cells were first surface-stained with anti-CD4, fixed, and then permeabilized by using CytoFix/Cytoperm (BD Biosciences). Fluorochrome-labeled mAbs to FoxP3 and IL-17A were from eBioscience, and IFN-γ mAb was from BD Biosciences.

Real-Time PCR. RNA was extracted from pancreatic tissue by using the RNeasy kit (Qiagen). RNA was reverse-transcribed by using Omniscript (Qiagen). Levels of Reg2 and PAP mRNA were measured by RT-PCR using gene expression assay primers from Applied Biosystems. Quantities of mRNA were calculated relative to the expression of the *Rpl7* housekeeping gene.

Cytokine ELISAs. Levels of IL-21 and IL-17 in culture supernatants were measured by using kits from R&D Systems. IFN-γ was measured by using a kit from BD PharMingen.

T Cell Proliferation and Suppression Assays. Treg functional activity was tested against responder $CD4^+CD25^-$ NOD/WT or NOD/*Il21r*-KO cells stimulated in the presence of anti-CD3 plus APCs. NOD/WT $CD4^+CD25^-$ responders and $CD4^+CD25^+$ Tregs from both NOD/WT and NOD/*Il21r*-KO mice were purified from total spleen by using magnetic microbeads (Miltenyi Biotec). $CD4^+CD25^-$ T cells (5×10^4) were cultured for 72 h in round-bottom 96-well plates with 5×10^4 APCs consisting of irradiated (3,000 rads) T cell-depleted splenocytes from NOD/WT or NOD/*Il21r*-KO mice, 1 μg/ml anti-CD3, and increasing numbers of $CD4^+CD25^+$ T cells. Proliferation was assessed by pulsing with 1 μCi of [^{3}H]thymidine for the last 8 h of culture, and incorporation was measured by liquid scintillation. Treg cells were unresponsive to stimulation with anti-CD3 plus APCs.

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1. Powers AC (2005) *Diabetes Mellitus* (McGraw–Hill, New York).
2. Anderson M, Bluestone J (2005) The NOD mouse: A model of immune dysregulation. *Annu Rev Immunol* 23:447–485.
3. Parrish-Novak J, *et al*. (2000) Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function. *Nature* 408:57–63.
4. Spolski R, Leonard WJ (2008) Interleukin-21: Basic biology and implications for cancer and autoimmunity. *Annu Rev Immunol* 26:57–79.
5. Noguchi M, *et al*. (1993) Interleukin-2 receptor γ chain mutation results in X-linked severe combined immunodeficiency in humans. *Cell* 73:147–157.
6. Zhou L, *et al*. (2007) IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nat Immunol* 8:967–974.
7. Korn T, *et al*. (2007) IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. *Nature* 448:484–487.
8. Nurieva R, *et al*. (2007) Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. *Nature* 448:480–483.
9. Coquet JM, *et al*. (2007) IL-21 is produced by NKT cells and modulates NKT cell activation and cytokine production. *J Immunol* 178:2827–2834.
10. Ozaki K, *et al*. (2002) A critical role for IL-21 in regulating immunoglobulin production. *Science* 298:1630–1634.
11. Zeng R, *et al*. (2005) Synergy of IL-21 and IL-15 in regulating $CD8^+$ T cell expansion and function. *J Exp Med* 201:139–148.
12. Kasaian MT, *et al*. (2002) IL-21 limits NK cell responses and promotes antigen-specific T cell activation: A mediator of the transition from innate to adaptive immunity. *Immunity* 16:559–569.
13. Ozaki K, *et al*. (2004) Regulation of B cell differentiation and plasma cell generation by IL-21, a novel inducer of Blimp-1 and Bcl-6. *J Immunol* 173:5361–5371.
14. Vinuesa C, *et al*. (2005) A RING-type ubiquitin ligase family member required to repress follicular helper T cells and autoimmunity. *Nature* 435:452–458.
15. Herber D, *et al*. (2007) IL-21 has a pathogenic role in a lupus-prone mouse model, and its blockade with IL-21R.Fc reduces disease progression. *J Immunol* 178:3822–3830.
16. Young DA, *et al*. (2007) Blockade of the interleukin-21/interleukin-21 receptor pathway ameliorates disease in animal models of rheumatoid arthritis. *Arthritis Rheum* 56:1152–1163.
17. King C, Ilic A, Koelsch K, Sarvetnick N (2004) Homeostatic expansion of T cells during immune insufficiency generates autoimmunity. *Cell* 117:265–277.
18. Yamanouchi J, *et al*. (2007) Interleukin-2 gene variation impairs regulatory T cell function and causes autoimmunity. *Nat Genet* 39:329–337.
19. Jain R, *et al*. (2008) Innocuous IFNγ induced by adjuvant-free antigen restores normoglycemia in NOD mice through inhibition of IL-17 production. *J Exp Med* 205:207–218.

20. Mensah-Brown E, Shahin A, Al-Shamisi M, Wei X, Lukic M (2006) IL-23 leads to diabetes induction after subdiabetogenic treatment with multiple low doses of streptozotocin. *Eur J Immunol* 36:216–223.
21. Vukkadapu S, *et al*. (2005) Dynamic interaction between T cell-mediated beta cell damage and beta cell repair in the run up to autoimmune diabetes of the NOD mouse. *Physiol Genomics* 21:201–211.
22. Narushima Y, *et al*. (1997) Structure, chromosomal localization, and expression of mouse genes encoding type III Reg, RegIII α, RegIII β, RegIII γ. *Gene* 185:159–168.
23. Unno M, *et al.* (2002) Production and characterization of Reg knockout mice: Reduced proliferation of pancreatic beta cells in Reg knockout mice. *Diabetes* 51(Suppl 3):S478–S483.
24. Berzins S, Venanzi E, Benoist C, Mathis D (2003) T cell compartments of prediabetic NOD mice. *Diabetes* 52:327–334.
25. Tritt M, Sgouroudis E, d'Hennezel E, Albanese A, Piccirillo C (2008) Functional waning of naturally occurring $CD4^+$ regulatory T cells contributes to the onset of autoimmune diabetes. *Diabetes* 57:113–123.
26. Salomon B, *et al*. (2000) B7/CD28 costimulation is essential for the homeostasis of the $CD4^+CD25^+$ immunoregulatory T cells that control autoimmune diabetes. *Immunity* 12:431–440.
27. Miljkovic D, *et al*. (2005) Interleukin-17 stimulates inducible nitric oxide synthase-dependent toxicity in mouse beta cells. *Cell Mol Life Sci* 62:2658–2668.
28. Gurr W, Shaw M, Li Y, Sherwin R (2007) RegII is a beta cell protein and autoantigen in diabetes of NOD mice. *Diabetes* 56:34–40.
29. Gurr W, *et al*. (2002) A Reg family protein is overexpressed in islets from a patient with new-onset type 1 diabetes and acts as T cell autoantigen in NOD mice. *Diabetes* 51:339–346.